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7. Reactivation of acetylcholinesterase activity and its therapeutic benefits in nerve agent intoxication

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Abstract. Organophosphorus chemical warfare nerve agents are potent inhibitors of the enzyme acetylcholinesterase (AChE). Inhibition of AChE at nerve terminals in peripheral tissues and the central nervous system (CNS) results in acetylcholine (ACh) overload and, depending on the extent of enzyme inhibition, cholinergic crisis. Current treatment strategies for nerve agent intoxication consist of using an oxime such as pyridine-2-aldoxime methylchloride (2-PAM) to reactivate the inhibited AChE and the anticholinergic drug atropine sulfate to antagonize the effects of

[1]Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, by the Institute of Laboratory Animal Resources, National Research Council. The research environment and protocols for animal experimentation were approved by the Institutional Animal Care and Use Committee (IACUC). The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The opinions or assertions contained herein are the private views of the authors, and are not to be construed as reflecting the views of the Department of the Army or the Department of Defense.

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excess ACh at muscarinic cholinergic receptors. In addition, a benzodiazepine, such as diazepam, is used to control the seizures. Atropine sulfate acts both peripherally and centrally to antagonize the action of ACh, but 2-PAM and all other currently available oximes (e.g., obidoxime, HI-6) act only in the peripheral tissues, because their quaternary charge limits entry into the CNS. The brain is a major target for the toxic effects of nerve agents. Inhibition of AChE in the brain results in seizures and neuropathology and contributes to the incapacitating behavioral and lethal effects of these agents. The inability of quaternary oximes to enter the brain and reactivate nerve agent-inhibited AChE is a major limitation of current oxime therapy. Protecting and restoring AChE activity in both the CNS and periphery is a major goal in the development of medical countermeasures against nerve agents. In this chapter, we analyzed and discussed the critical relationship between oxime reactivation and *in vivo* protection by oximes. We also provided experimental evidence of AChE activity reactivation in the CNS, in particular by a tertiary oxime, and its relationship to therapeutic efficacy in acute nerve agent exposure.

Abbreviations

ACh	:	acetylcholine
AChE	:	acetylcholinesterase
ChE	:	cholinesterase
CNS	:	central nervous system
DAM	:	diacetylmonooxime
im	:	intramuscular
ip	:	intraperitoneal
LD_{50}	:	median lethal dose
MINA	:	monoisonitrosoacetone
OP	:	organophosphorus compound
2-PAM	:	pyridine-2-aldoxime methylchloride
PR	:	protective ratio
RBC	:	red blood cell
sc	:	subcutaneous

Introduction

The potential for exposure to organophosphorus (OP) nerve agents exists on the battlefield, as a terrorist threat to civilian populations and as an occupational health hazard to workers demilitarizing outdated chemical warfare weapons. The agents of greatest concern along with their chemical names and two-letter military designation are tabun (*o*-ethyl *N,N*-dimethyl phosphoramidocyanidate; GA), sarin (isopropyl methylphosphonofluoridate; GB), soman (pinacolyl methylphosphonofluoridate; GD), cyclosarin (cyclohexyl

methylphosphonofluoridate; GF), VX (O-ethyl S-(2-diisopropylamino ethyl methyl phosphonothioate) and the Russian V-type compound designated VR (*O*-isobutyl S-(2-diethylamino)ethyl methylphosphonothioate). These agents are extremely potent inhibitors of the cholinesterase (ChE) enzymes, which include acetylcholinesterase (AChE) and butyrylcholinesterase. Their toxic effects are due to hyperactivity of the cholinergic system as a result of inhibition of ChE, in particular, AChE, and the subsequent increase in the concentration of the neurotransmitter acetylcholine (ACh) in the brain and periphery (1-3). Exposure causes a progression of toxic signs, including hypersecretions, muscle fasciculations, tremors, convulsive seizures, respiratory distress and death (3,4). A combined regimen of prophylaxis and therapy is the most effective medical countermeasure for dealing with the threat of nerve agent poisoning to military personnel (4-7). Pretreatment with carbamate ChE inhibitors, such as pyridostigmine bromide, shields a fraction of ChE in the periphery from irreversible inhibition by the nerve agents (8,9). In the event of nerve agent poisoning, immediate therapeutic treatment with an oxime, such as 2-PAM (pyridine-2-aldoxime methylchloride; pralidoxime), obidoxime (Toxogonin®) or HI-6 (1-(4-carbamoylpyridino) methoxymethyl-2-(hydroxyiminomethyl) pyridinium), is used to reactivate any unaged, inhibited AChE (3,10). Administration of an anticholinergic drug, such as atropine sulfate, antagonizes the effects of excess ACh at muscarinic receptor sites (4,7).

Atropine sulfate has been universally adopted as the optimal anticholinergic therapy, but countries vary as to their choice of AChE reactivator. Commercially available oximes for OP poisoning refer to compounds that comprise an oxime moiety (R-CH=NOH) attached to a quaternary nitrogen pyridinium ring. They reactivate OP-inhibited ChE by dephosphonylating the enzyme active site *via* interaction with a nearby anionic subsite. Reactivation occurs through nucleophilic attack by the oxime on the phosphorous atom, splitting an oxime-phosphonate away from the active site. The regenerated esteratic site is subsequently able to bind and cleave its normal substrate, ACh. This action of the oximes is considered to be the major mechanism of their antidotal action in reversing the toxic/lethal effects of OP nerve agents (11,12). The recovery of functional AChE activity is the most critical benefit of all nerve agent antidotal actions.

2-PAM chloride is the oxime currently used in the U. S. for the emergency treatment of OP nerve agent exposure. Some countries use different salts (e.g., methanesulfonate, iodide) of 2-PAM. Other countries prefer bis-pyridinium compounds such as obidoxime (Toxogonin®), trimethoxime (TMB-4) or HI-6 as oxime antidotes (4,13). Although 2-PAM provides adequate protection against the nerve agents sarin and VX (14), it is less effective against other

nerve agents (e.g., tabun, soman, cyclosarin) (15,16). In recent years, several oximes, such as MMB-4 (methoxime; 1,1'-methylenebis[4-[(hydroxyimino) methyl]pyridinium]), HLö7 (1-[[[4-(aminocarbonyl)pyridinio]methoxy]methyl] -2,4-bis[(hydroxyimino)methyl] pyridinium), HI-6, and ICD585 (1-(4-amino-carbonylpyridinio)-3(2-hydroxyiminomethylpyridinio)-propane dichloride monohydrate), have been found to possess much better antidotal capacity than 2-PAM in response to nerve agent intoxication in animal studies (13,17-26).

Oximes currently used as medical countermeasures (e.g., 2-PAM, obidoxime, HI-6) have quaternary structures that are very similar, differing only by the number of pyridinium rings and by the position of the oxime moiety on the ring (Figure 1). Quaternary oximes are positively charged compounds, so they cannot cross the blood-brain barrier and their action is limited to only the periphery. The inability of quaternary oximes to enter the

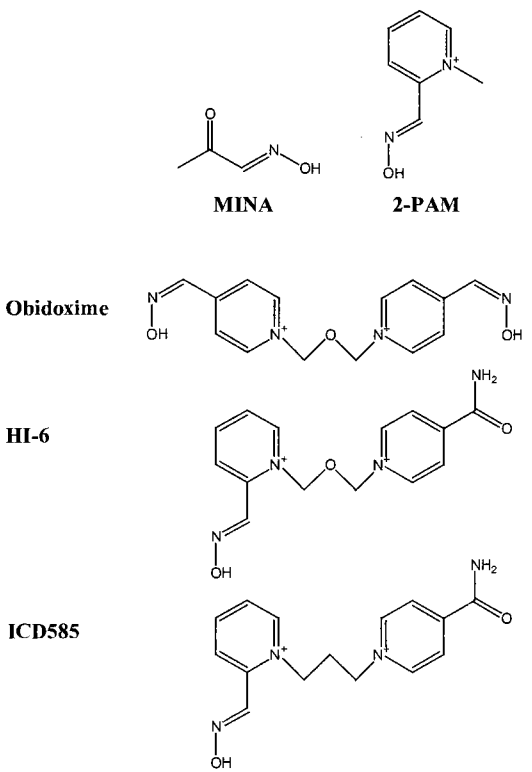

Figure 1. Structures of oximes.

brain prevents them from reactivating nerve agent-inhibited AChE within the central nervous system (CNS) and is, therefore, a major limitation of current oxime therapy.

The brain is a major target of OP nerve agents. Inhibition of AChE in the brain results in prolonged seizure activity and neuropathology, thus contributing further to the incapacitating and lethal effects of these agents (27,28). The influence of central AChE on protection is reinforced by the reports showing that partially protecting AChE in the CNS with reversible ChE inhibitors, such as physostigmine, huperzine-A, or galantamine, prior to nerve agent exposure could improve survival, reduce seizure activity and neuropathology, and lessen behavioral incapacitation following nerve agent exposure (29-33). It is, therefore, thought by many medical chemical defense scientists that oxime reactivation of nerve agent-inhibited AChE in the CNS would provide significant benefits (34-36). However, there have been no systematic studies to investigate this concept.

Monoisonitrosoacetone (MINA) and diacetylmonooxime (DAM) are tertiary oximes that had been investigated in the 1950's. Both are highly lipid soluble and readily penetrate the blood-brain barrier (37) to reactivate AChE within the CNS (37,38). The ability of MINA and DAM to reactivate inhibited AChE in the CNS could have significant impact in the treatment of the toxic effects of nerve agents. Indeed, when used alone or in combination with atropine sulfate, MINA and DAM were shown to raise the LD_{50} doses of sarin in several animal species (38-43). Unfortunately, these two tertiary oximes were not pursued further, due to reports that quaternary pyridinium oximes (e.g., 2-PAM) were more potent reactivators of phosphorylated AChE by several orders of magnitude in human erythrocytes (see review of 44).

In this chapter, we discuss the relationship between *in vitro* AChE reactivation by oximes and *in vivo* oxime protection in nerve agent intoxication. Furthermore, we discuss the roles of peripheral and CNS oxime reactivation of nerve agent-inhibited AChE in improving survival and reducing or eliminating other CNS sequelae (seizures and neuropathology) of OP nerve agent intoxication in an *in vivo* guinea pig model.

The relationship between oxime reactivation and *in vivo* oxime protection

Since the discovery that mono-pyridinium oximes were effective reactivators of OP-inhibited AChE (10), a number of mono-pyridinium and bis-pyridinium oximes have been synthesized and tested for their efficacy against OP compounds, but no oxime has been identified that is effective against all OP nerve agents (13,23,45). A number of different oximes, such as

2-PAM, obidoxime (Toxogonin®), or HI-6, have been adopted by different countries for treatment of OP poisoning (4,7). Regardless of whether efficacy was evaluated by *in vitro* or *in vivo* investigations (46,47), structure-activity relationships for oxime efficacy are poorly understood (45,48) because oxime reactivation has a complex dependency on the nucleophilicity and orientation of the oxime as well as on the structure of the OP-AChE conjugate (49,50).

The difficulty in establishing a correlation between oxime structure, AChE reactivation and oxime protection has led some authors to suggest that mechanisms other than AChE reactivation may be responsible for *in vivo* oxime protection against nerve agents (51-54). However, recent analyses of oxime efficacy with improved parameters for oxime reactivation have indicated a strong correlation between *in vitro* oxime reactivation and *in vivo* oxime protection against VX, sarin, VR and cyclosarin (11,12,26). The group of oximes evaluated in these studies (shown in Fig. 1) consisted of 2-PAM and obidoxime, which are licensed for treatment of nerve agent poisoning, HI-6, which is being considered for regulatory approval, and ICD585, which has been considered for advanced development (7,26). As a consequence of their success in the drug development process, a more extensive database existed for these oximes than for other less efficacious oximes.

To eliminate the complications that interspecies variation would have introduced into the analysis, only oxime protection data from guinea pigs were used for current analysis because guinea pigs contain low levels of plasma carboxylesterase, a characteristic that they share with humans (55). *In vivo* oxime protection was expressed as a protective ratio (PR), which is the ratio of the nerve agent LD_{50} in oxime/atropine sulfate-treated animals divided by the nerve agent LD_{50} in untreated animals. The presence of high levels of plasma carboxylesterase (56) in mice and rats precludes a comparison of PR values generated in mice and rats with PR values generated in guinea pigs. The variations in plasma carboxylesterase create variable LD_{50} values for nerve agents across species that lead to variation in the PR values for an oxime in different species (57). *In vitro* oxime reactivation was expressed as a second-order rate constant (k_{r2}), where k_{r2} was calculated by the equation $k_{r2} = k_r/K_D$ from the dissociation constant of the inhibited AChE-oxime complex (K_D) and the maximal first-order rate constant for oxime reactivation (k_r). Oxime reactivation was described by k_{r2} because the product of k_{r2} and oxime concentration [Ox] describes the rate of oxime reactivation at the concentrations of oxime that are usually observed *in vivo* (i.e., $[Ox] < K_D$).

Table 1 is a compilation of PR values describing the protection provided by atropine sulfate and oximes against nerve agents in guinea pigs. The dose

of atropine sulfate (46-50 μmol/kg) in all studies was a dose that maximizes protection when used in conjunction with oximes in guinea pigs and was sufficient to control the cholinergic effects of nerve agents and to terminate nerve agent-induced seizures (58). The doses of obidoxime in Table 1 (40-56 μmol/kg) are lower than the doses for 2-PAM, HI-6 and ICD585 (130-145 μmol/kg) because obidoxime is more toxic (23). *In vivo* oxime protection against nerve agents as measured by PR values varied 29-fold from 2.6 to 76 among the nerve agent/oxime combinations. *In vivo* protection by 2-PAM or obidoxime against nerve agents varied 14-fold, whereas protection by HI-6 and ICD585 varied only 2.5-fold and 3.7-fold, respectively. In general, the PR values for each oxime against nerve agents decreased in the order VX ≥ sarin > VR > cyclosarin, although there are examples (e.g., HI-6 against VX and sarin; ICD585 against VR and cyclosarin) where this order was reversed.

Table 2 is a compilation of k_{r2} values for oxime reactivation of AChE inhibited by different nerve agents. Oxime reactivation expressed as k_{r2} varied >500-fold (0.026 to 14.3 $mM^{-1}min^{-1}$) among the nerve agent/oxime combinations. The values for k_{r2} varied 127-fold for 2-PAM and 212-fold for

Table 1. Comparison of *in vivo* oxime protection against nerve agents in guinea pigs.

Nerve agent	Protective ratio for oxime (PR)[a]			
	HI-6	ICD585	Obidoxime	2-PAM
VX	66	47.6	58	36.8
Sarin	76	33.3	59	22.7
VR	39.8	12.7	13.6	6.28
Cyclosarin	31.1	19.1	4	2.6

[a]PR values, where PR = (agent LD_{50} in drug-treated group)/(agent LD_{50} in saline-treated group), were determined in guinea pigs that received oxime and atropine (im) 1 min after receiving nerve agent (sc). Atropine doses were 46-50 μmol/kg, and oxime doses were 130-145 μmol/kg for 2-PAM, HI-6 and ICD585 and 40-56 μmol/kg for obidoxime. PR values were taken from Dawson (23) and Maxwell et al. (26).

Table 2. Comparison of *in vitro* reactivation rate constants for oximes against nerve agent-inhibited AChE from guinea pigs.

Nerve agent	k_{r2} for oxime (mM^{-1} min^{-1})[a]			
	HI-6	ICD585	Obidoxime	2-PAM
VX	0.14	0.11	12.5	3.3
Sarin	0.33	0.24	14.3	2.7
VR	0.14	0.091	0.19	0.10
Cyclosarin	0.19	0.045	0.059	0.026

[a]Rate constants were determined at pH 7.4 and 37 °C. Values for k_{r2} were taken from Maxwell et al. (26) and Worek et al. (60).

obidoxime and generally decreased in the order VX ≥ sarin > VR > cyclosarin. Oxime reactivation by HI-6 and ICD585 varied only 2.4-fold and 5.3-fold, respectively, between the highest and lowest k_{r2} values.

A regression analysis of the relationship between the *in vivo* protection by oximes and their reactivation of nerve agent-inhibited AChE is shown in Figure 2. Protection by oximes against the toxicity of VX, sarin, VR and cyclosarin in guinea pigs was expressed as PR-1 because a PR of 1 denotes an absence of oxime protection. Oxime reactivation was normalized for different doses of oxime by multiplying k_{r2} by the dose of oxime ([Oxime]) used to determine the PR values in guinea pigs. The points representing the values for PR-1 and k_{r2}· [Oxime] for each nerve agent/oxime combination separated into two populations that are described by two offset but parallel lines. One data set describes oxime efficacy by 2-PAM and obidoxime, and the second data set describes oxime efficacy by HI-6 and ICD585. No points in the HI-6/ICD585 data overlapped into the 95% confidence boundaries of the 2-PAM/obidoxime data set or vice versa, which suggested that these data sets described different relationships between oxime reactivation and oxime protection. Inasmuch as the difference between the two y-intercepts on this Log_{10}-Log_{10} plot was 0.83 the mean linear displacement between these two populations of data points was $10^{0.83}$ = 6.8. This indicated that HI-6/ICD585 achieved 6.8 times greater *in vivo* protection than did 2-PAM/obidoxime for a given degree of oxime reactivation across a range of oxime doses.

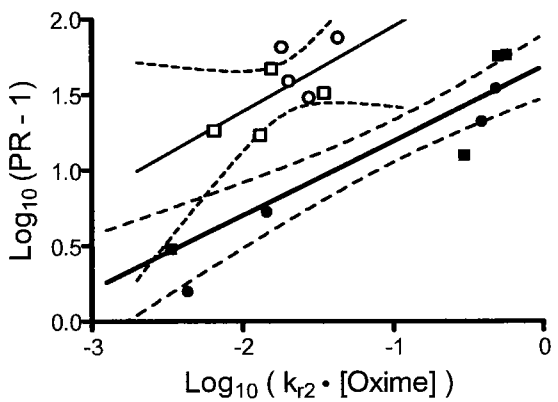

Figure 2. Regression analysis of oxime protection with oxime reactivation. Data points for HI-6 (○), ICD585 (□), 2-PAM (●) and obidoxime (■) were taken from Table 1 and Table 2. 95% confidence limits are indicated by (---). PR-1 = PR of 1, which denotes an absence of oxime protection.

If oxime reactivation is assumed to be the sole mechanism of oxime protection, then HI-6 and ICD585 were 6.8 times more effective in coupling oxime reactivation to oxime protection (i.e., survival) than were 2-PAM and obidoxime. This study confirmed previous observations that HI-6 is a better reactivator than the other oximes in the study, but it also demonstrated that there is an advantage of HI-6 and ICD585 compared to 2-PAM and obidoxime that is separate from any differences between these pairs of oximes in their ability to reactivate OP-inhibited AChE. This advantage appears to be unrelated to any direct pharmacological effect of HI-6 or ICD585 (51) because there is no correlation between the rank order of oxime protection observed in this study and the rank order of the ability of oximes to produce direct pharmacological effects at either the neuromuscular junction (52) or in the CNS (53). While there is no obvious mechanism at this time for the enhanced oxime protection provided by HI-6 and ICD585, our study does indicate that the enhanced protection may be due to the isonicotinamide group that is common to both HI-6 and ICD585 and is absent from 2-PAM and obidoxime (see Fig. 1). This isonicotinamide group was originally incorporated into the structure of bis-pyridinium oximes to reduce their toxicity (59), but our study suggests that it may also improve the efficacy of oximes. Inasmuch as these structure-activity relationships were identified from oxime efficacy data restricted to guinea pigs, the extrapolation of these relationships to other species, such as humans, should be done with caution because oxime reactivation of AChE in guinea pigs and in humans has been reported to differ significantly (60).

CNS reactivation of AChE and therapeutic benefits in nerve agent poisoning

Inhibition of AChE by OP nerve agents in the brain can result acutely in prolonged seizures (*status epilepticus*) and neuropathology, which in turn contribute to the incapacitating and lethal effects of these agents (27,28). We have long been interested in the concept of oxime reactivation of nerve agent-inhibited AChE in the CNS since, in theory, this should reverse and/or prevent the CNS consequences of nerve agent intoxication. To test this hypothesis, we have conducted preliminary studies with the tertiary oxime MINA in guinea pigs to determine its ability to reactivate sarin-inhibited AChE activity in blood, peripheral tissues and brain, its efficacy against the lethal effects of sarin intoxication, and its ability to prevent/terminate sarin-induced seizures and neuropathology.

Effect of tertiary oxime MINA on sarin-inhibited AChE

When blood and tissue samples were taken 60 min after 1 x LD_{50} of sarin followed 15 min later by administration of MINA, at the time of maximal AChE inhibition by sarin (61), the tertiary oxime MINA reactivated sarin-inhibited AChE in the brain regions (brainstem, cerebellum, cortex,

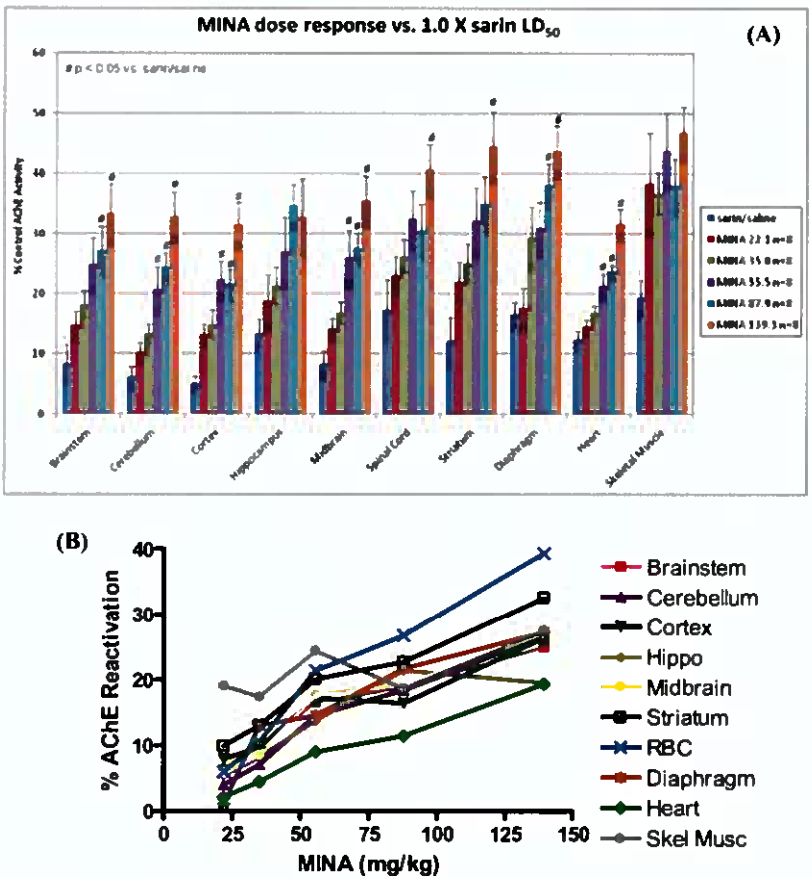

Figure 3. Dose-dependent effects of MINA (A) and % reactivation by MINA (B) of brain and peripheral tissue AChE activity inhibited by sarin in guinea pigs. MINA (22.1, 35.0, 55.5, 87.9 or 139.3 mg/kg, im) injected 15 min after sarin (1 x LD_{50}, sc) exposure. Animals euthanized and tissues removed 60 min after sarin for AChE analysis. RBC = red blood cell.

hippocampus, midbrain, spinal cord, and striatum), blood, and peripheral tissues (diaphragm, heart, and skeletal muscle) with tissue specificity and in a dose-dependent manner. Figure 3A shows the higher AChE activity, when compared with vehicle (saline-saline) treatment, in brain regions and some peripheral tissues following various doses of MINA in sarin-intoxicated guinea pigs. The percentage of AChE reactivation over that of sarin-inhibited AChE activity in various tissue compartments and red blood cell (RBC) is shown in Figure 3B, whereas the percentage of AChE reactivation for the highest dose of MINA (139.3 mg/kg) is tabulated in Table 3, in comparison with that for 2-PAM (25 mg/kg).

Effect of CNS AChE reactivation on survival

Having confirmed earlier reports (37,38) that MINA reactivates both peripheral and CNS AChE inhibited by the nerve agent sarin, we examined whether the capacity of MINA to reactivate CNS AChE would make it more efficacious than the quaternary oxime 2-PAM against lethal nerve agent exposure. Our previous experiments (62) showed that while 2-PAM was an effective reactivator of peripheral AChE inhibited by sarin, unlike MINA, it was ineffective in reactivating sarin-inhibited AChE in the brain (Table 3).

To determine the efficacy of MINA against the lethal effects of nerve agent exposure, we generated 24-hr sc LD_{50} estimates for sarin in guinea pigs treated im 1 min post-exposure with atropine sulfate (0.5 mg/kg), 2-PAM (25 mg/kg), atropine sulfate + 2-PAM, MINA (35 or 60 mg/kg), atropine

Table 3. Brain and peripheral tissue reactivation of sarin-inhibited AChE by 2-PAM and MINA.

Tissue	% AChE Reactivation	
	2-PAM (25 mg/kg)[a]	MINA (139.3 mg/kg)[a]
Brainstem	0.0	25.0
Cerebellum	1.2	26.7
Cortex	4.5	26.4
Hippocampus	0.0	19.5
Midbrain	0.0	27.2
Striatum	0.0	32.5
Red Blood Cells	42.0	39.3
Diaphragm	35.3	27.3
Heart	45.8	19.3
Skeletal Muscle	31.8	27.5

[a]2-PAM administered 5 min and MINA 15 min after sarin challenge.

sulfate + MINA, or atropine sulfate + 2-PAM + MINA. All of the treatment groups and sarin challenge levels were run in parallel and randomized to the animals. Between 26 -36 animals were used to generate each LD_{50} estimate at 24 hr by probit analysis. The doses of atropine sulfate and 2-PAM used in this study were human relevant doses of these drugs. The atropine sulfate dose was the human equivalent of three 2-mg atropine autoinjectors based on body surface area scaling formulas, and the 2-PAM dose was the human equivalent of three 600-mg 2-PAM autoinjectors on a mg/kg basis in a 70-kg individual. The MINA doses used (35.0 and 60 mg/kg, im) were those that resulted in approximately 10-15% reactivation respectively of regional brain AChE (Figure 3B). The 24-hr LD_{50} values and the PR based on atropine sulfate therapy are shown in Table 4.

The results show that MINA was more effective than atropine sulfate treatment alone or 2-PAM treatment alone against sarin intoxication. In combination with atropine sulfate, MINA at a dose of 60 mg/kg was 2.4 times more effective than atropine sulfate + 2-PAM. MINA was most effective when added to atropine sulfate + 2-PAM treatment. In these treatment groups, the LD_{50} of sarin increased 4.3- to 5.1-fold compared to that in the atropine sulfate + 2-PAM groups.

Table 4. Efficacy of MINA treatment on 24-hr survivability after sarin intoxication.

Treatment (mg/kg)[a]	N	24-hr LD_{50} μg/kg (95% CI)	LD_{50} Ratio[b]	Statistical significance
ATR (0.5)	26	40.2 (37.7 – 42.8)	1.0	
2-PAM (25)	29	82.3 (50.8 -133.5)	2.0	
ATR + 2-PAM	32	132.9 (89.9 -196.6)	3.3	$p<0.05$ vs. ATR
MINA (35)	31	142.9 (124.7 -163.8)	3.6	$p<0.05$ vs. ATR or 2-PAM
MINA (60)	28	292.6 (231.3 – 370.2)	7.3	$p<0.05$ vs. ATR, 2-PAM, or ATR+2-PAM
ATR + MINA (35)	30	175.5 (117.7 -262.1)	4.4	$P<0.05$ vs. ATR or 2-PAM
ATR + MINA (60)	28	313.7 (210.8 – 466.9)	7.8	$p<0.05$ vs. ATR, 2-PAM or ATR+2-PAM
ATR + 2-PAM + MINA (35)	36	670.3 (254.2 – 1767.8)	16.7	$p<0.05$ vs. ATR+ 2-PAM or ATR+MINA (35)
ATR + 2-PAM + MINA (60)	30	572.5 (369.1- 888.1)	14.2	$p< 0.05$ vs. ATR+2-PAM or ATR+MINA (60)

[a]Administered im 1 min after sc sarin challenge. ATR = atropine sulfate.
[b]Protective ratio = LD_{50} of oxime-treated/LD_{50} of ATR-treated animals.

These results clearly demonstrate the benefit of reactivating CNS AChE with an oxime on survival outcomes following sarin intoxication. Surprisingly, MINA treatment alone without any atropine sulfate was more effective than atropine sulfate alone, 2-PAM alone, or the combination of atropine sulfate + 2-PAM. Additionally, MINA alone was just as effective as MINA + atropine sulfate treatment. The most dramatic benefit from the use of MINA occurred when it was used in combination with atropine sulfate + 2-PAM treatment. In these 2 treatment groups, approximately 10-15% reactivation of CNS AChE in concert with the peripheral AChE reactivation of both 2-PAM and MINA and a small dose of atropine sulfate (0.5 mg/kg, im) resulted in large increases in survival (Table 4).

Effect of CNS AChE reactivation on seizure activity and neuropathology

Epileptic seizure activity is a prominent effect of OP nerve agents on the CNS. It is generally agreed that nerve agent-induced seizures are triggered by the rapid rise in ACh in sensitive limbic brain areas subsequent to inhibition of AChE in these areas by the nerve agent (27,63,64). This increase in excitatory activity initiates seizure activity that rapidly progresses to *status epilepticus* if not counteracted with an anticonvulsant treatment. Research from many laboratories has shown that only three classes of drugs demonstrate the ability to stop nerve agent-induced seizures: anticholinergics, such as scopolamine, benactyzine and caramiphen (65,66); benzodiazepines, such as diazepam, midazolam, and lorazepam (67-69); and antagonists of the N-methyl-d-aspartate subtype of the glutamate receptor, such as MK-801 (dizocilpine), TCP (thienylphencyclidine), and ketamine (70-74). Since the inhibition of CNS AChE is the event that sets the conditions for these seizures to develop, we reasoned that a centrally acting oxime like MINA should block seizure development by reactivating sarin-inhibited AChE in critical brain structures. To this end, we performed two experiments using a validated guinea pig nerve agent-seizure model that has successfully tested multiple anticonvulsant drugs (28,65,69,75,76). The first study demonstrated the dose-dependent ability of MINA to prevent sarin-induced seizures. The second, a time-course study, showed that a high dose of MINA was capable of stopping sarin-induced seizures even when administered at varying times after seizure onset.

Adult male Hartley guinea pigs were prepared with cortical stainless screw electrodes to record brain electroencephalographic (EEG) activity using standard small animal surgical procedures (69,76). They were allowed to recover for one week and were challenged with 2 x LD_{50} (84 μg/kg, sc) sarin and treated 1 min after exposure with atropine sulfate (0.5 mg/kg, im).

For the dose-dependent study, groups of animals then received MINA, immediately after atropine sulfate, at a dose of 18, 32, 42.2 or 56 mg/kg, im, or 2-PAM at a dose of 18, 32 or 56 mg/kg, im. In the second study, the animals also received 2-PAM (25 mg/kg, im) at the same time (i.e., 1 min after sarin) they received the atropine sulfate, since animals that received atropine sulfate alone succumbed within 2-5 min to the lethal effects of sarin without developing seizures. Groups of animals were treated at 5, 10, 20 or 40 min after the onset of EEG seizure activity with 56 mg/kg, im, MINA, a dose of MINA that prevented seizures in all test animals in the dose-response experiment. After treatment, the animals were observed, and EEG was recorded continuously over the next five hours. The occurrence and duration of seizure activity were determined from the EEG record by two independent observers. In animals that survived overnight EEG were again recorded for 30 min at 24 hr after exposure. These survivors were weighed, deeply anesthetized (75 mg/kg, ip, pentobarbital), and then perfused transcardially with saline followed by 10% formalin. The brains were paraffin processed, sectioned at 5 μm and stained with hematoxylin and eosin (H&E) for histopathology evaluation. Histopathology was qualitatively graded, using a previously published procedure (28,65), by an individual who was not aware of the treatment of a given subject. The basolateral amygdala was evaluated and used to represent the magnitude of brain injury since this brain region in guinea pigs is particularly sensitive to nerve agent-induced damage (65).

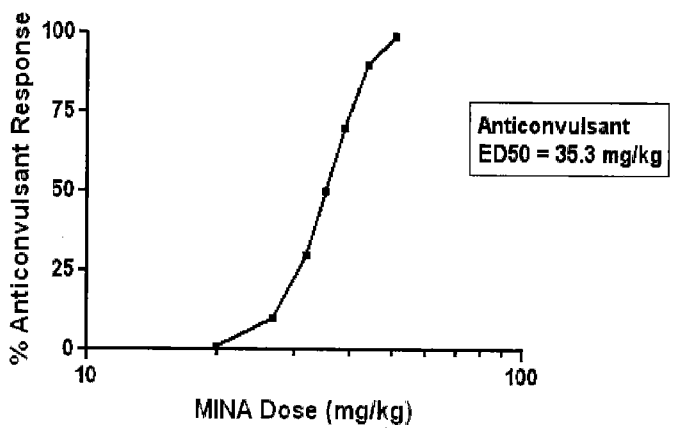

Figure 4. Anticonvulsant dose-dependent effect curve for MINA when administered 1 min after sarin (2 x LD_{50}, sc) challenge. Note the X-axis is in log units.

In the first study, MINA was capable of preventing or rapidly stopping sarin-induced seizures in a dose-dependent manner (Figure 4). All animals treated with 18 mg/kg MINA developed seizures, while seizures were prevented in 33, 83 and 100% of the animals treated with 32, 42.2 and 56 mg/kg MINA, respectively (N=6/dose group). This resulted in an anticonvulsant ED_{50} = 35.3 (95% confidence limits = 24.8-43.3) mg/kg MINA as determined by probit analysis. In most cases in which effective doses of MINA were used, seizures never developed. However, in three cases (2 at 32 mg/kg MINA, 1 at 42.2 mg/kg MINA) seizure activity developed briefly, but stopped spontaneously after a short period (30 and 47 sec, respectively, for the two animals treated with 32 mg/kg MINA; 7 min 28 sec for the animal treated with 42.2 mg/kg MINA); these animals were considered treatment successes. Of the 11 animals treated with MINA that developed seizures, 8 of these animals failed to survive for 24 hr (5 at 18 mg/kg MINA; 3 at 32 mg/kg MINA), while only one (32 mg/kg MINA dose group) of the 13 animals in which MINA prevented or stopped seizures failed to survive 24 hr. Animals in which MINA prevented or stopped seizures lost an average of 15.5 g, equivalent to 4.6% of pre-exposure body weight, while those in which seizures were not prevented lost an average of 26.8 g, equivalent to 7.8% of pre-exposure body weight.

All animals treated with 2-PAM, regardless of dose, developed seizures (average seizure onset time = 6 min 53 sec after sarin exposure). Of the 15 animals treated with 2-PAM (18 mg/kg, N=3; 32 mg/kg, N=6; 56 mg/kg, N=6) 5 animals did not survive 24 hr (1 at 18 mg/kg; 3 at 32 mg/kg; 1 at 56 mg/kg), for a survival rate of 66%. The 10 animals treated with 2-PAM that did survive lost an average of 58 g, equivalent to 17.6 % of pre-exposure body weight. An analysis of variance of these post-exposure body weight changes showed that MINA-treated animals lost significantly less weight ($F_{2, 23}$ = 30.68, $p < 0.001$), regardless of whether seizures were prevented or not, than did 2-PAM-treated animals.

In the time-course study, 56 mg/kg MINA was capable of terminating sarin-induced seizures in a time-dependent fashion. Treatment at 5 min after seizure onset was 100% (6 of 6) successful in stopping seizures, while at 10, 20 and 40 min after seizure onset the success rate dropped progressively to 83% (5 of 6), 50% (3 of 6) and 17% (1 of 6), respectively. Figure 5 displays the inverse relationship between successful control of seizures and seizure termination latencies as a function of treatment delay. The earlier MINA was administered, the more likely the seizure was to be stopped and stopped rapidly after MINA injection; the later MINA was administered, the less likely it was to stop the seizure, and seizure termination was more protracted. As in the first study, severity of body weight loss and incidence of brain

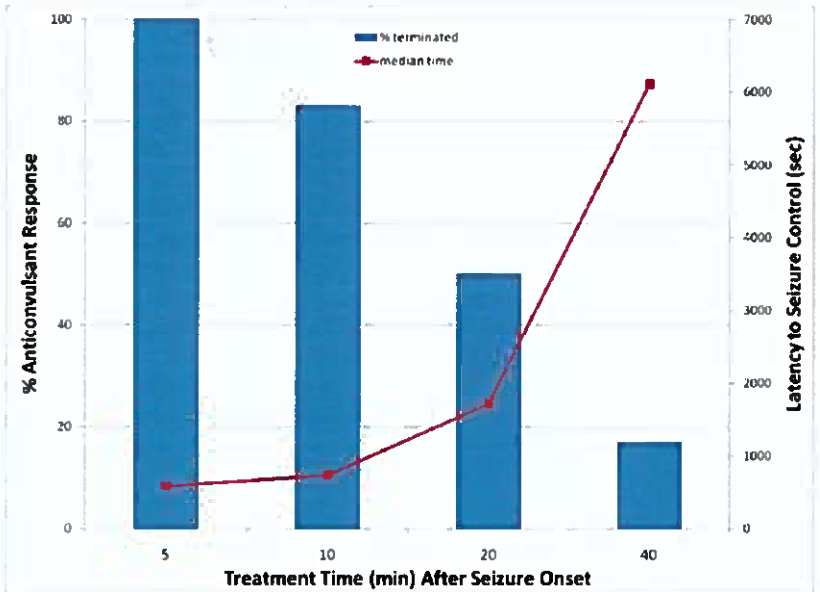

Figure 5. The relationship between successful control of seizures and seizure termination latencies as a function of treatment delay. Time-dependent reduction in the anticonvulsant effect (represented by blue bars) and latencies for seizure termination (represented by red line) from the injection of MINA (56 mg/kg) as a function of the delay between sarin-induced seizure onset and MINA treatment. Each bar represents % anticonvulsant response; the horizontal line points represent group medians. N = 6/group.

pathology were highly dependent upon seizure control. Animals in which seizures were stopped by MINA treatment lost significantly less body weight (N = 15; mean weight loss = 11. 5 g) in the 24 hr after sarin exposure than did those animals in which seizures were not controlled (N = 7; mean weight loss = 39.4 g). A Kruskal-Wallis one way analysis of variance on ranks showed that the percentage body weight loss by the animals in which seizures were stopped by MINA treatment (3.4%) was significantly less ($H = 7.38$, $df = 1$, $p = 0.007$) than the percentage body weight loss by the animals in which seizures were not stopped by MINA (15%).

The capacity of MINA to reactivate sarin-inhibited AChE in the CNS and to stop seizures was strongly associated with its ability to prevent seizure-related neuropathology. Animals that had seizure activity terminated by MINA treatment (N = 15) displayed no neuropathology. Four of the

animals in the first study in which seizures were not prevented by MINA treatment and five of the seven surviving animals in the second study in which seizures were not stopped by MINA displayed extensive neuropathology. The two animals that were free of neuropathology from the second study displayed normal EEGs on the 24-hr record, but were judged to be still seizing at the end of the recording session on the day of exposure because both displayed repetitive low amplitude, high frequency, bursts that had been associated with return of seizure activity in other studies (75). Table 5 displays the neuropathology data collapsed across both studies and shows a high degree of association between the development of neuropathology and the failure of a treatment to stop seizure activity. Microscopic examples of this neuropathology are displayed in Figure 6. In 2-PAM–treated animals, brain tissue from 9 of the 10 survivors was available for histology, and all 9 animals displayed substantial evidence of neural damage in the examined brain areas (Figure 6).

Seizure prevention and/or termination and the subsequent reduction of seizure-induced neuropathology were also strongly associated with CNS reactivation of sarin-inhibited AChE with MINA. A MINA dose of 56 mg/kg resulting in 15-20% reactivation of regional brain AChE was sufficient to block or counteract seizure activity produced by sarin and prevent neuropathology. MINA was effective in 100% of the animals when injected 1 to 5 min after seizure onset and in 50% of the animals when injected as late as 20 min after seizure onset. In all of the animals in which seizure was prevented or terminated, neuropathology was greatly reduced or prevented. In the time course study, one may question why MINA became progressively less effective in controlling sarin-induced seizures as the time for treatment was systematically delayed. This is unlikely due to "ageing" of the sarin-inhibited enzyme, since the half-time for this process is on the order of hours (77,78). In contrast, seizure resistance could be predicted from a previously published neuropharmacological model of nerve agent-induced seizures (27). In this model, while seizure onset is dependent upon inhibition of CNS AChE activity by the nerve agent (i.e., a cholinergic initiating event), once seizures

Table 5. Relationship between the numbers of animals displaying neuropathology as a function of the ability of MINA or 2-PAM to stop seizure activity.

	Neuropathology	
Seizure	No	Yes
Off	18	2
On	0	27
Chi Square = 35.67, df = 1, $p < 0.001$		

related neuropathology. Control of seizures also resulted in an attenuated body weight loss that has been prominently associated with nerve agent seizure activity in experimental animal models. Based on the data from the reactivation study, it can be speculated that 10-15% reactivation of brain AChE, a percentage associated with a 35-mg/kg dose of MINA, was sufficient to enhance survival following supra-lethal doses of sarin. This dose of MINA by itself as a treatment was more effective than either atropine or 2-PAM and was just as effective as the combination of atropine plus 2-PAM treatment (Table 4). The most dramatic effect of MINA on survival occurred when it was combined with atropine and 2-PAM. The PR increased from 3.3 to nearly 17.

Summary and conclusions

Oximes are true antidotes for OP nerve agent intoxication in that they reverse the effect of the nerve agent on the AChE enzyme. Current clinically used oximes are quaternary in structure and do not reactivate AChE inhibited in the CNS. The present data with the tertiary oxime MINA show that reactivation of inhibited CNS-AChE can provide significant benefits in preventing or reversing the neuro-toxicological effects of nerve agents. While the development of an effective, centrally active oxime may be possible, several scientific and regulatory issues will provide challenges. Among the oximes developed to date, the ability of individual oximes to reactivate AChE inhibited by different nerve agents significantly varies, such that, as yet, no "universal oxime" provides high levels of protection against all nerve agents. This same issue will no doubt also be a challenge to any centrally active oxime that is developed. In all probability, an oxime that effectively treats the widest number of nerve agents, not necessarily all the nerve agents, may be a reasonable compromise. A centrally active oxime by definition will enter the brain. Because of this, the side-effect profile of such a compound will become an important factor when considering development. Ultimately, the ideal oxime will have a wide safety margin between the dose that is necessary to reactivate nerve agent-inhibited AChE and the dose that produces untoward neurological and/or behavioral effects.

Oximes play a critical role in the treatment of OP nerve agent intoxication. Arguably they are the most important component of nerve agent treatment regimen because they can restore the activity of AChE. The other components (atropine sulfate and anticonvulsant drug) provide only supportive benefit and facilitate the effectiveness of the oxime. The relationship analysis of *in vitro* and *in vivo* data suggests that reactivation of nerve agent-inhibited AChE is the primary mechanism by which oximes

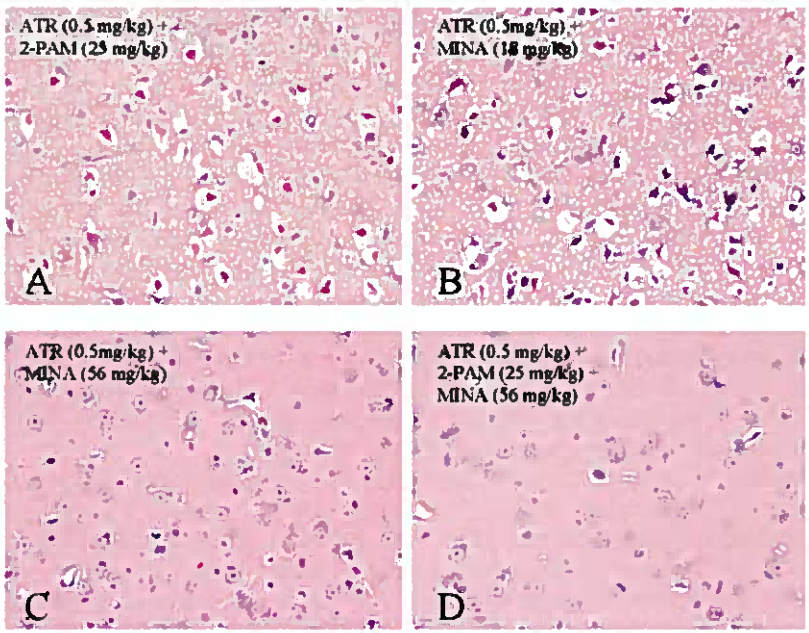

Figure 6. Representative light micrographs of basolateral amygdala from sarin-exposed guinea pigs. (A) ATR and 2-PAM or (B) ATR and MINA (18 mg/kg) given at one min after sarin exposure did not prevent brain pathology (characterized by vacuolization and scattered red or dark pyknotic neurons). In contrast, (C) ATR and MINA at a higher dose (56 mg/kg) were effective in preventing brain injury. Brain damage was also prevented when (D) MINA at 56 mg/kg was given at 20 min after the onset of seizure as an adjunctive treatment to ATR and 2-PAM.

develop the seizure activity itself recruits other neurotransmitter systems, most notably the glutamatergic system, to reinforce seizure activity. Over time, the glutamate system exerts progressively greater control of the seizure such that simply reversing the inhibition of CNS AChE is not capable by itself of stopping seizure activity initiated by cholinergic transmission. However, the capacity of MINA to terminate seizures (Figure 5) and prevent brain damage (Figure 6D) when given up to 20 min after seizure onset suggests that cholinergic mechanisms are still actively involved.

The results of these studies clearly show the benefit of oxime reactivation of centrally inhibited AChE in the treatment of sarin intoxication. Reactivation of CNS AChE with MINA contributed significantly to enhanced survival, prevention or termination of seizures and prevention of seizure-

exert their efficacy. Furthermore, those oximes that are the most efficient in reactivating inhibited AChE are also the most effective in treating the lethal effects of nerve agent intoxication. Our results demonstrate that oxime effectiveness is significantly enhanced if nerve agent-inhibited AChE in the CNS is also reactivated. The benefits of a centrally acting oxime are not only limited to improving survival, but also important in preventing and/or terminating seizures and preventing seizure-related neuropathology. Further research and development is needed to identify other oximes that can readily penetrate the CNS. A centrally active oxime will be most effective if it is able to reactivate AChE inhibited by a broad spectrum of nerve agents.

Acknowledgments

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